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920522-905378

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE THE APPLICATION OF)
) Examiner:
Schacht et al.)
) Group Art Unit No.
SERIAL NO.: 10/009,808)
)
FILED: December 7, 2001)
)
FOR: Functional Poly-Alpha-Aminoacid)
Derivatives Useful For The Modification)
Of Biologically Active Materials And)
Their Use)

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CLAIM FOR PRIORITY

Honorable Director of Patents and Trademarks
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Dear Sir:

Under the International Convention, for the purposes of priority, applicant claims the benefit of European Application No. 99870125.4, filed June 17, 1999.

A certified copy of said application is appended hereto.

August 18, 2003

Respectfully submitted,

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BE00/00066

Bescheinigung Certificate Attestation

28/12 4

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

99870125.4

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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I.L.C. HATTEN-HECKMAN

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 99870125.4
Demande n°:

Anmeldetag:
Date of filing: 17/06/99
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
UNIVERSITEIT GENT
9000 Gent
BELGIUM

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Title of the invention:
Titre de l'invention:
End group functionalized poly alpha amino acid derivatives

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

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Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE/TR
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Bemerkungen:
Remarks:
Remarques:

END GROUP FUNCTIONALIZED POLY ALPHA AMINO ACID DERIVATIVES

End group functionalized poly-α-amino acid derivatives

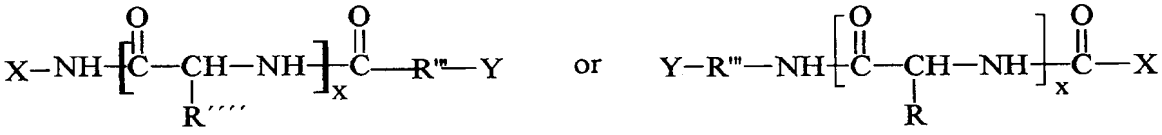
Background

In the past decades there has been a great interest in the use of endgroup functionalized poly ethylene glycol for the modification of peptides, proteines, enzymes and non-peptidic drugs. Abochowski described a method for the modification of proteines with polyethylene glycol grafted onto amino side groups along the proteine.
It was shown that polyethylene glycol grafted proteines exhibit a longer plasma half life in vivo, were less immunogenic and rendered proteines and enzymes more thermostable.
Zalipsky, Harris, Hooftman and others have reviewed a variety of methods for introducing reactive groups at the chain end of polyethylene glycol which can react in a selective manner with proteine side group functionalities such as amino groups, thiols, guanidines....

In biomaterial science, grafting of polymer material surfaces with polyethylene glycol chains has been extensively described as a method for improving surface biocompatibility. Surface peg-ylation can be achieved by chemical grafting of polyethylene glycol onto a preformed surface as well as by applying polymers having polyethylene glycol as constructive part of their backbone or alternatively as grafted side groups. Such polymers can be used as core material or be applied as surface coating.

Polyethylene glycol is a rather stable polymer and is not subjective to enzymatic or hydrolytic degradation under physiological conditions.

The present invention describes a method for preparing water soluble poly α-amino acids and derivatives having a reactive group at one or both ends of a linear polymer chain.
The reactive endgroups can be an amine, alcohol, carboxyl group, reactive ester, reactive carbonate ester, a thiol, a thiol precursor such as a disulfide, a maleinimide, an acrylate, methacrylate, acrylamide, methacrylamide or an other vinylgroup suitable for radical, anionic or cationic polymerization.
If the poly α-amino acid or its derivative is composed of L-amino acid units, such polymer will be substrate for enzymatic degradation. However poly α-amino acids and derivatives composed of D-amino acids will be stable towards peptide degrading enzymes



With x ranging from 1 to 2.000, typically 1 to 500.

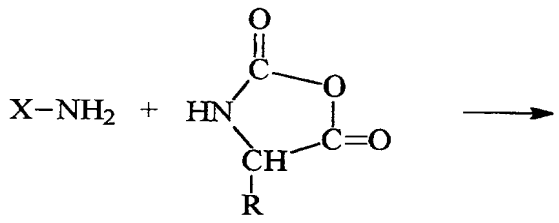
The commonly used synthesis of poly[N^5-(2-hydroxy-ethyl)-L-glutamine] (PHEG) is performed by polymerization of N-carboxy-anhydride of γ-benzyl-L-glutamate[ ], followed by aminolysis of poly-γ-benzyl-L-glutamate with large excess of 2-amino ethanol in presence of 2-hydroxypyridine as a bifunctional catalyst[ ]. Aminolysis of the benzyl function is usually accompanied by extensive chain scission, due to cleavage of the backbone amide bonds[ ].

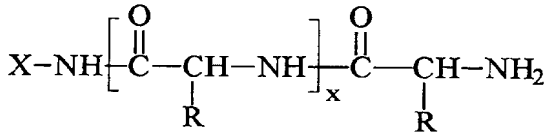
The monomers used in this invention are N-carboxy-anhydrides of γ-methyl, γ-benzyl or γ-trichloroethylesters of L-glutamate[ ] and L-aspartate.
Initiation of their polymerization with bifunctional initiators containing primary amino group or termination of the polymerization with bifunctional terminating agent leads to derivatives with a reactive group at one end of the polymer chain. A combination of initiation and termination with suitable reagents gives polymers with reactive groups at both ends of the linear polymer chain.
The termination reaction proceeds without forming of a substituted pirrolidonering as it is in the case of polymerization of N-carboxyanhydride of γ-benzyl-L-glutamate initiated by primary amines.

Aminolysis of poly-γ-trichloroethyl-L-glutamate and poly-γ-trichloroethyl-L-aspartate is performed with 2-aminoethyanol (2-fold excess) or 2,3-dihydroxy-propylamine (2-fold excess) in presence of catalytic quantities of 2-hydroxypyridine. The aminolysis proceeds without chain scission.
These mono- and bifunctional soluble poly α-aminoacids are prepared also by activated monomer mechanism of polymerization of N-carboxyanhydrides using appropriate initiating and terminating agents.

Poly(α-amino acid) derivatives with either one or two functional end groups can be prepared via ring opening polymerization of N-carboxyanhydride derivatives of the α-amino acid (derivatives). Polymerization can occur via the primary amine-initiated ring opening process (route 1) or via the activated monomer process (route 2).
End group functionalities are introduced either in the initiation step and/or the termination step and/or the chemical modification of a chain end group.

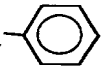
Route 1 to endgroup functionalized polymers :





with R = $(CH_2)n-\overset{O}{\overset{\|}{C}}-OR'$

n = 1, 2

R' = CH_3, CH_2-  , CH_2CCl_3

X = A-NH-R" A = protective group : e.g. $-\overset{O}{\overset{\|}{C}}-O-CH_2$- 

R" = $(CH_2)_n$, n = 2-20
aryl, aryl alkyl

A-S-R" A = protective group

$-S$-  , trityl, ...

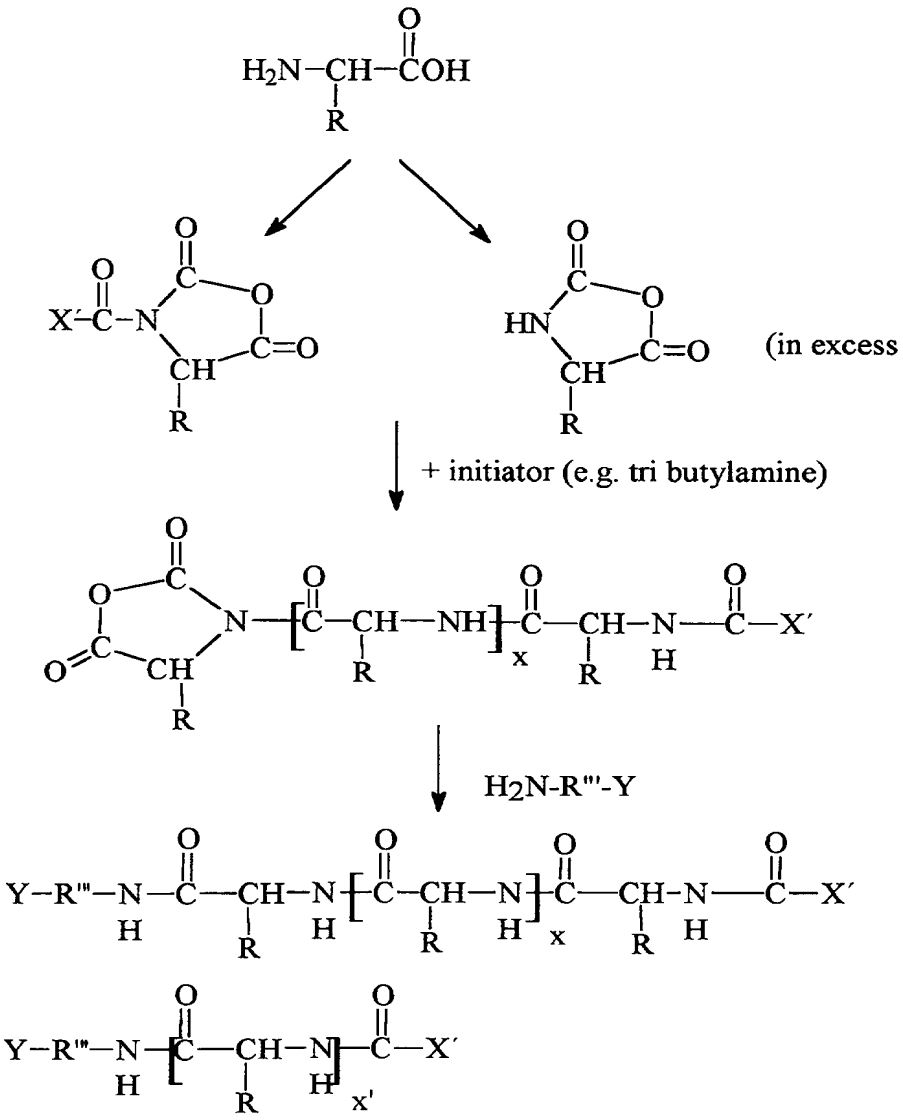
A-O-R" or

$A-\overset{O}{\overset{\|}{C}}-R"$

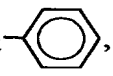
A = protective group

e.g. tetrahydropyranyl, ...

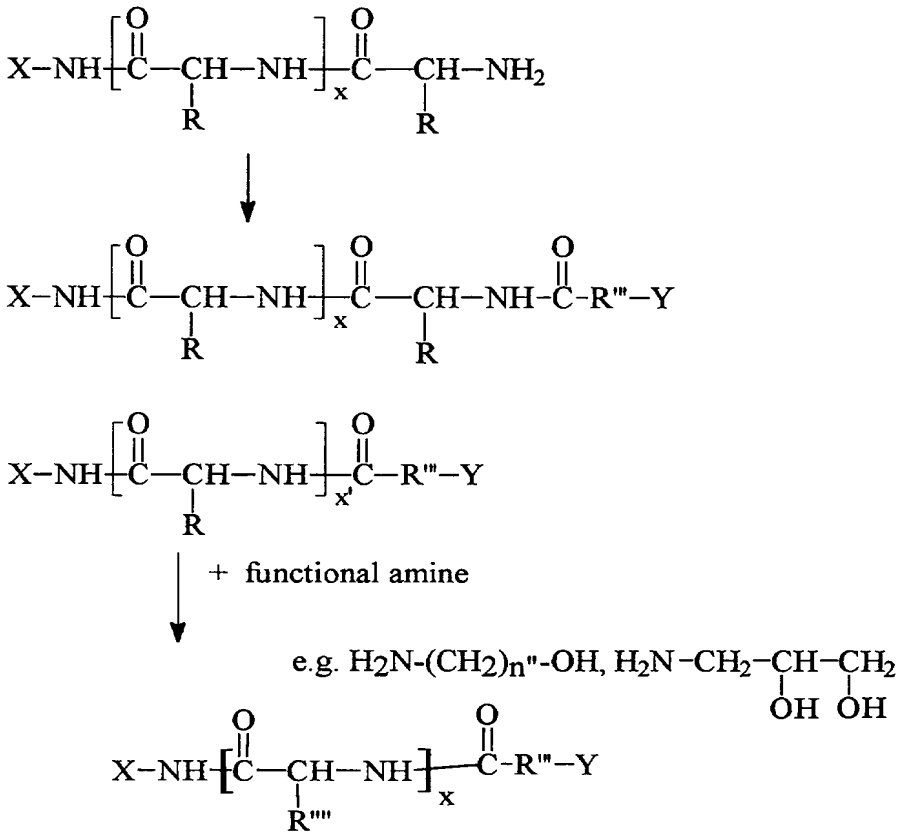
Route 2 to endgroup functionalized polymers :



with R = $(CH_2)n-\overset{O}{\overset{\|}{C}}-O-R'$ n = 1, 2

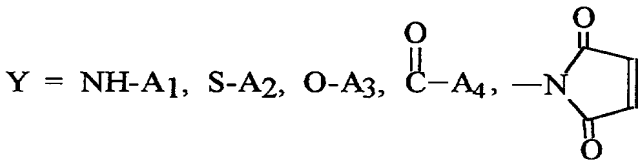
R' = CH_3, CH_2-  , CH_2CCl_3

x´ is 1 to 2000, typically 500

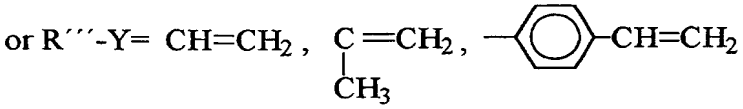


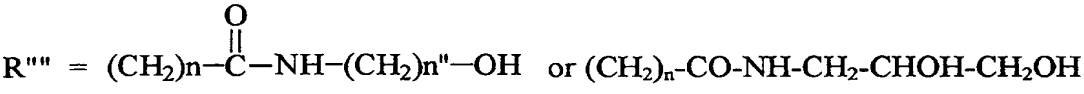
With $R''' = (CH_2)_{n'}$ $n' = 2\text{-}20$

Aryl, alkyl aryl,



A = suitable protective group for amine (A_1), thiol (A_2), alcohol (A_3), carboxyl group (A_4)





EXAMPLES :

SYNTHESIS OF PHEG's (and PHEA's) WITH FUNCTIONAL END GROUPS

1. Synthesis of trichloroethyl ester of poly-L-glutamic acid

N-carboxyanhydride of trichloroethyl glutamate (TCEG-NCA) (1g) is dissolved in 10 ml dry 1,2-dichloroethane. The solution is cooled down to 10°C. Triethylamine (0,010 g, 5 mol %) is added to the solution of NCA. The reaction is followed by IR spectroscopy. After the end of the polymerisation the solution is precipitated in pentane, the product is isolated by filtration and dried under vacuum. The polymer is characterised by ^{1}H NMR (DMF-d_7). The molecular weight is determined by ^{1}H NMR and GPC (polystyrene standards, THF as eluent). M_n = 20000. Yield 99 %.

2. Synthesis of poly-[N-(2-hydroxyethyl)-L-glutamine] with functional end group

Poly-[N-(2-hydroxyethyl)-L-glutamine] (PHEG) with functional end group(s) is synthesised by initiation of the polymerisation of TCEG-NCA with suitable initiators or by termination the reaction with suitable terminating agents.

2.1. Synthesis of poly-[N-(2-hydroxyethyl)-L-glutamine] with amino end group

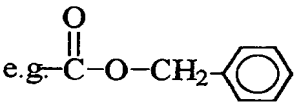
2.1.1. Polymerisation of N-carboxyanhydride of γ-trichloroethyl-L- glutamate by initiation with 1-triphenylmethylaminoethylamine

N-Carboxyanhydride of γ-trichloroethyl-L- glutamate (TCEG-NCA) (2 g) is dissolved in 20 ml dry 1,2-dichloroethane. The solution is cooled down to 10°C.

1-Triphenylmethylaminoethylamine (0,099 g, 5 mol % to TCEG-NCA) is dissolved in 2 ml 1,2-dichloroethane and added to the solution of NCA. After the end of the polymerisation, determined by IR spectroscopy, 3-fold molar excess of acetic anhydride and equimolar quantity of triethylamine are added and the reaction mixture is stirred for another 2 h at room temperature. The solution is precipitated in pentane and the polymer is isolated by filtration and drying under vacuum.

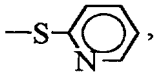
The molecular weight is determined by ^{1}H NMR (DMF-d_7) and GPC (polystyrene standards, THF as eluent). M_n = 6000. Yield 99 %.

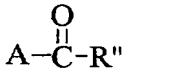
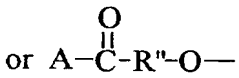
X′ = A-NH-R" or A-NH-R"-O-

A = protective group : e.g. 

R" = $(CH_2)_n$, n = 2-20
aryl, alkyl aryl

A-S-R" or A-S-R"-O-

A = protective group
 , trityl, ...

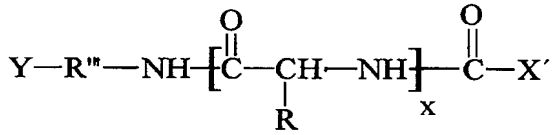
A-O-R"
or A-O-R"-O-

or 

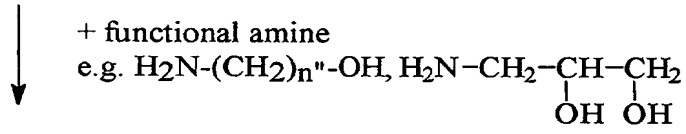
A = protective group
e.g. tetrahydropyranyl, ...

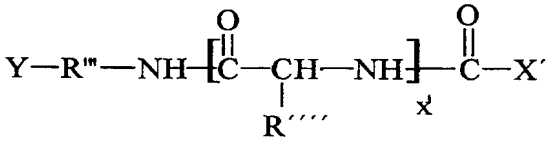
Y = R"-NH-A
R"-O-A

R"-S-A

A = suitable protective group

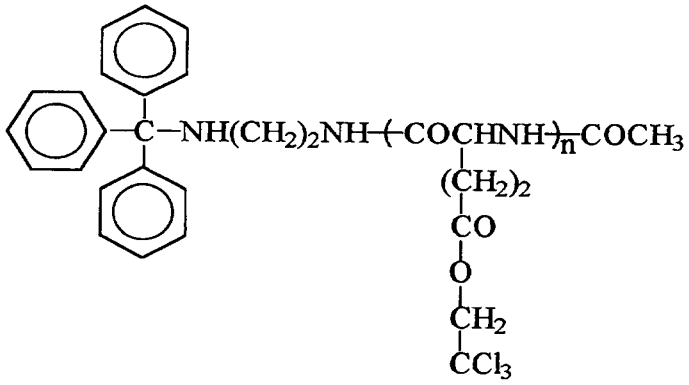






Where R′′′′ and R′′′ may be the same as for route 1

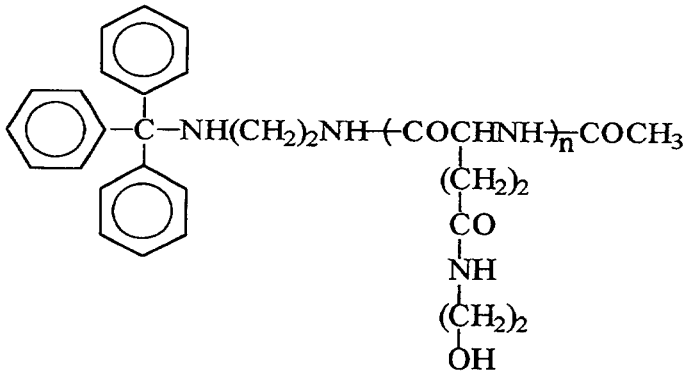
^{1}H NMR (DMF-d_7) analysis confirms the following structure of the polymer:



2.1.2. Aminolysis of trichloroethyl ester of poly-L-glutamic acid (2.1.1) with ethanolamine

1 g (3,8 mmol units) of the polymer is dissolved in 10 ml dry N,N-dimethylformamide. The solution is cooled down to10°C and 0,69 ml (11,5 mmol) ethanolamine and 0,36 g (3,8 mmol) 2-hydroxypyridine are added. The reaction is followed by IR spectroscopy. After the end of the aminolysis, the polymer is isolated by precipitation in ether, filtration and drying under vacuum. The product is purified by gel filtration on Sephadex G-25 (water as eluent) and isolated by lyophilization. The polymer is characterised by ^{1}H NMR (D_2O) and GPC (dextran standards, water as eluent). M_n = 4000. Yield 95 %.

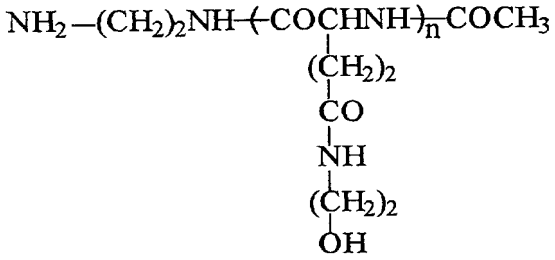
^{1}H NMR analysis confirms the following structure of the polymer:



2.1.3. Deprotection of poly-[N-(2-hydroxyethyl)-L-glutamine] (PHEG) with triphenyl-methyl end group (2.1.2)

1 g polymer is dissolved in 10 ml trifluoroacetic acid and stirred at room temperature for 0,5 h. Trifluoroacetic acid is removed by evaporation under vacuum. The polymer is dissolved in water and centrigugated. The supernatant is purified by gel filtration on Sephadex G-25 (water as eluent). The product is isolated by lyophilization.

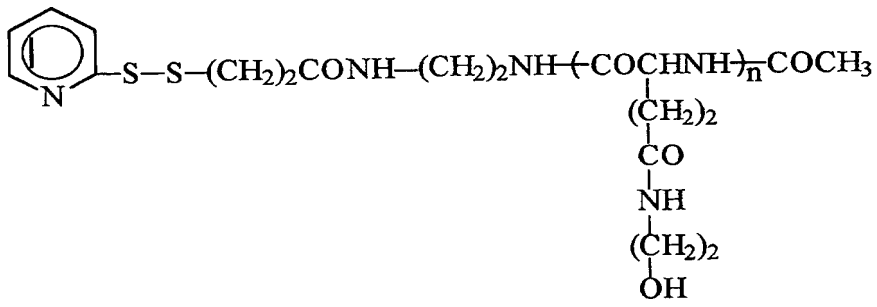
^{1}H NMR (D_2O/DCl) analysis confirms the following structure of the polymer:



2.2. Synthesis of poly-[N-(2-hydroxyethyl)-L-glutamine] with SS-Py end group

The polymer from 2.1.3 (1 g) is dissolved in 0,1 M phosphate buffer, pH 7,5 (100 ml). N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, 0,6 g) is dissolved in 30 ml ethanol and added to the solution of the polymer. After 2 h reaction at room temperature, the mixture is separated on Sephadex G-25 (water as eluent). PHEG-SPDP is isolated by lyophilization.

^{1}H NMR (D_2O) analysis confirms the following structure of the polymer:



The concentration of pyridyldithio groups is determined also in the presence of 0,1 M DTT, using ε = 8080 $M^{-1}cm^{-1}$ at 343 nm for released pyridine-2-thione.

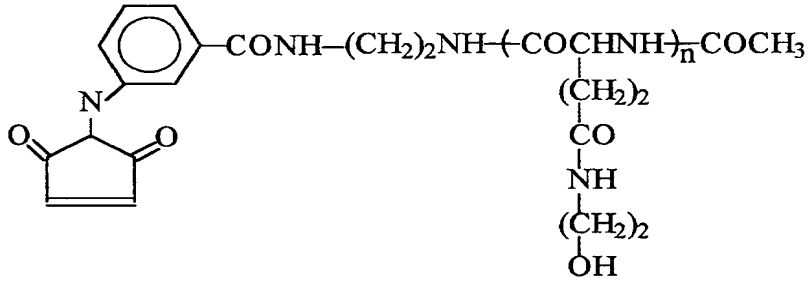
2.3. Synthesis of poly-[N-(2-hydroxyethyl)-L-glutamine] with thiol end group

Poly-[N-(2-hydroxyethyl)-L-glutamine] with SS-Py end group (PHEG-SPDP) is dissolved in 0,1 M acetate buffer, pH 4,5, containing 0,1 M NaCl (10 mg/ml), and dithiothreitol (DTT) is added to give concentration of 10mM. After 30 min at room temperature, the DTT-treated mixture is desalted into 0,1 M sodium phosphate buffer, pH 7,2, containing 1mM ethylenediaminetetraacetic acid (EDTA). The number of thiol groups generated is determined with 5,5'-dithiobis(2-nitrobenzoic acid).

2.4. Synthesis of poly-[N-(2-hydroxyethyl)-L-glutamine] with maleimide end group

The polymer from 2.1.3 (1 g) is dissolved in 0,1 M phosphate buffer, pH 7,0 (200 ml). m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, 0,2 g) is dissolved in 10 ml N,N-dimethylformamide and added to the solution of the polymer. After stirring at room temperature for 1 h, the mixture is separated on Sephadex G-25 (water as eluent) and the polymer is isolated by lyophilization.

^{1}H NMR (D_2O) analysis confirms the following structure of the polymer:

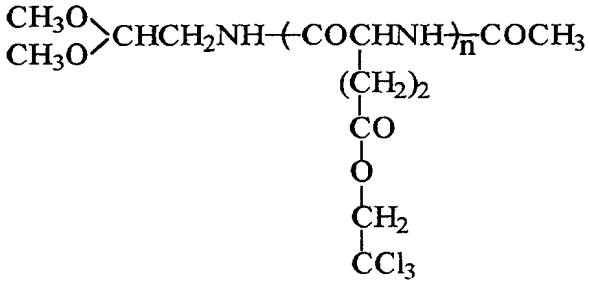


2.5. Synthesis of poly-[N-(2-hydroxyethyl)-L-glutamine] with aldehyde end group

2.5.1. Polymerisation of N-carboxyanhydride of γ-trichloroethyl-L-glutamate by initiation with aminoacetaldehyde-dimethylacetal

The polymerisation is initiated by aminoacetaldehyde-dimethylacetal. The reaction is carried out and the product is isolated and characterised as described in 2.1.1 . Yield 99 %.

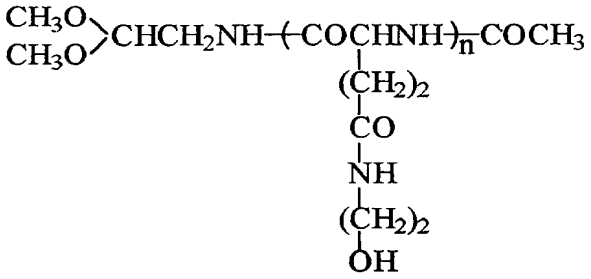
^{1}H NMR (DMF-d_7) confirms the following structure of the polymer obtained:



2.5.2. Aminolysis of trichloroethyl ester of poly-L-glutamic acid (2.5.1) with ethanolamine

The aminolysis is carried out and the product is isolated and characterised as it is described in 2.1.2). Yield 99 %.

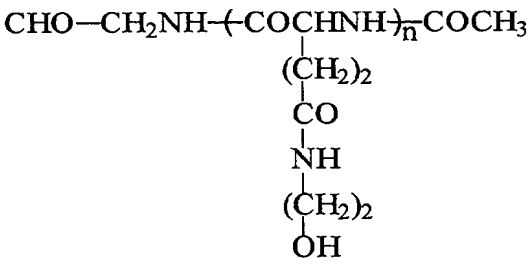
^{1}H NMR (D_2O) confirms the following structure of the polymer obtained:



2.5.3. Hydrolysis of the acetal groups to aldehyde group

The polymer from 2.5.2 (1 g) is dissolved in 10 ml 3 % hydrochloric acid and stirred at room temperature for 1 h. The mixture is separated on Sephadex G-25 and the polymer is isolated by lyophilization.

^{1}H NMR (D_2O) and determination of the aldehyde groups by reaction with hydroxylamine hydrochloride confirm the following structure of the polymer obtained:



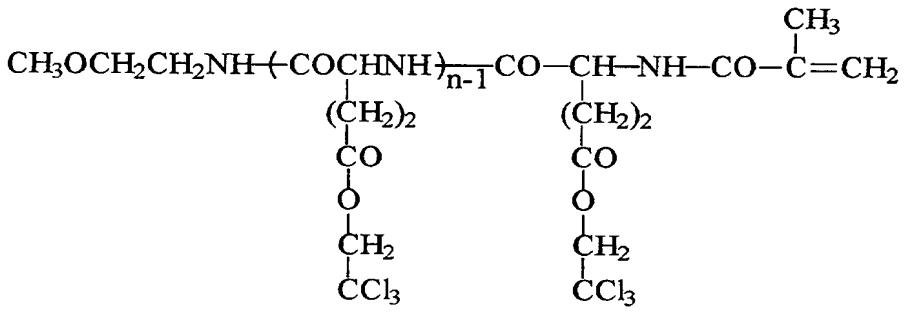
2.6. Synthesis of poly-[N-(2-hydroxyethyl)-L-glutamine] with methacrylate end group

2.6.1. Polymerisation of N-carboxyanhydride of γ-trichloroethyl-L- glutamate by initiation with 2-methoxyethylamine and termination with methacrylic anhydride

TCEG-NCA (2 g) is dissolved in 20 ml dry 1,2-dichloroethane. The solution is cooled down to 10°C. 2-Methoxyethylamine (0,023 g, 5 mol % to TCEG-NCA) is dissolved in 2 ml 1,2-dichloroethane and added to the solution of NCA. After the end of the polymerisation, determined by IR spectroscopy, 3-fold molar excess of methacrylic anhydride and equimolar quantity of triethylamine are added and the reaction mixture is stirred for another 2 h at room temperature. The solution is precipitated in pentane and the polymer is isolated by filtration and drying under vacuum.

The molecular weight is determined by ^{1}H NMR (DMF-d_7) and GPC (polystyrene standards, THF as eluent). M_n = 6500. Yield 99 %.

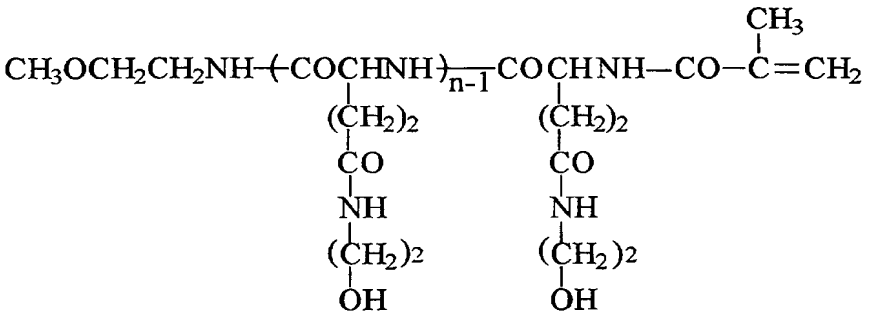
^{1}H NMR (DMF-d_7) analysis confirms the following structure of the polymer:



2.6.2. Aminolysis of trichloroethyl ester of poly-L-glutamic acid (2.6.1) with ethanolamine

The aminolysis is carried out and the product is isolated and characterised as it is described in 2.1.2). Yield 99 %.

^{1}H NMR (D_2O) confirms the following structure of the polymer obtained:

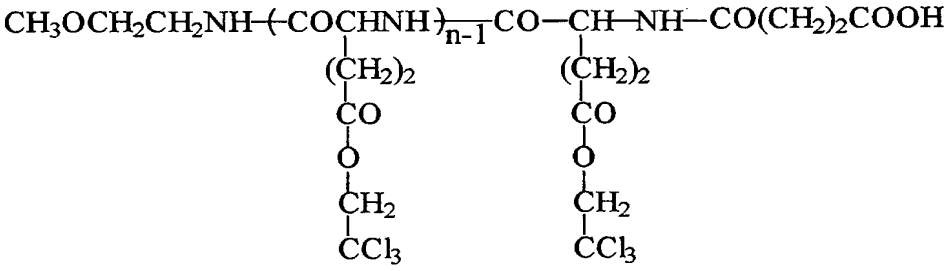


2.7. Synthesis of poly-[N-(2-hydroxyethyl)-L-glutamine] with carboxylic end group

2.7.1. Polymerisation of N-carboxyanhydride of γ-trichloroethyl-L- glutamate by initiation with 2-methoxyethylamine and termination with succinic anhydride

TCEG-NCA (2 g) is dissolved in 20 ml dry 1,2-dichloroethane. The solution is cooled down to 10°C. 2-Methoxyethylamine (0,023 g, 5 mol % to TCEG-NCA) is dissolved in 2 ml 1,2-dichloroethane and added to the solution of NCA. After the end of the polymerisation, determined by IR spectroscopy, 3-fold molar excess of succinic anhydride and equimolar quantity of triethylamine are added and the reaction mixture is stirred for 24 h at room temperature. Then 1,2 g citric acid is dissolved in 50 ml water and added to the reaction mixture. The polymer is extracted with 1,2-dichloroethane, the solution is dried over $MgSO_4$ and precipitated in pentane. The polymer is isolated by filtration and drying under vacuum. Yield 95 %.

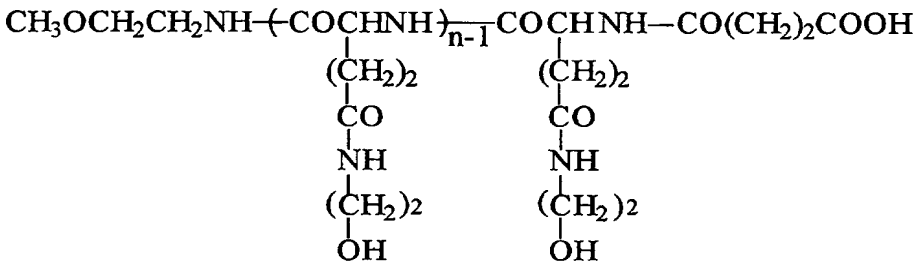
^{1}H NMR (DMF-d_7) confirms the following structure of the polymer obtained:



2.7.2. Aminolysis of trichloroethyl ester of poly-L-glutamic acid (2.7.1) with ethanolamine

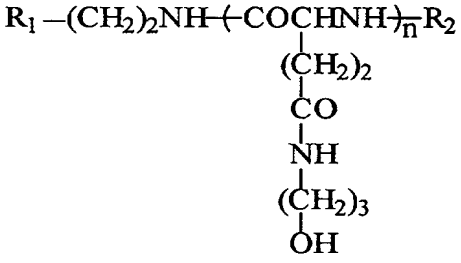
The aminolysis is carried out and the product is isolated and characterised as it is described in 2.1.2). Yield 98 %.

^{1}H NMR (D_2O) confirms the following structure of the polymer obtained:



3. Synthesis of poly-[N-(3-hydroxypropyl)-L-glutamine] with functional end group

Poly-[N-(3-hydroxypropyl)-L-glutamine] (PHPG) with functional end group(s) is synthesised by initiation of the polymerisation of TCEG-NCA with suitable initiators or by termination the reaction with suitable terminating agents as described in 2.1.1, 2.4.1 and 2.5.1. The aminolysis reaction is carried out with 3-amino-1-propanol as described in 2.1.2. After deprotection (as in 2.1.3) or hydrolysis (as in 2.4.3) the following polymers are obtained:



$R_1 = NH_2$, $R_2 = COCH_3$

R_1 = SS–Py, $R_2 = COCH_3$

R_1 = maleimide, $R_2 = COCH_3$

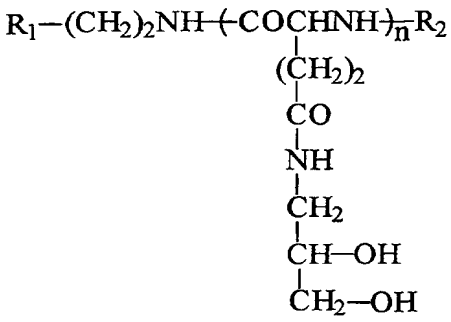
$R_1 = CHO$, $R_2 = COCH_3$

$R_1 = CH_3O$, $R_2 = CH(CH_3)=CH_2$

$R_1 = CH_3O$, $R_2 = COOH$

4. Synthesis of poly-[N-(2,3-dihydroxypropyl)-L-glutamine] with functional end group

Poly-[N-(2,3-dihydroxypropyl)-L-glutamine] (PDHPG) with functional end group(s) is synthesised by initiation of the polymerisation of TCEG-NCA with suitable initiators or by termination the reaction with suitable terminating agents as described in 2.1.1, 2.4.1 and 2.5.1. The aminolysis reaction is carried out with 3-amino-1,2-propandiol as described in 2.1.2. After deprotection (as in 2.1.3) or hydrolysis (as in 2.4.3) the following polymers are obtained:



$R_1 = NH_2$, $R_2 = COCH_3$

$R_1 = SS{-}Py$, $R_2 = COCH_3$

$R_1 =$ maleimide, $R_2 = COCH_3$

$R_1 = CHO$, $R_2 = COCH_3$

$R_1 = CH_3O$, $R_2 = CH(CH_3){=}CH_2$

$R_1 = CH_3O$, $R_2 = COOH$

5. Synthesis of trichloroethyl ester of poly-L-aspartic acid

N-carboxyanhydride of trichloroethyl aspartate (TCEA-NCA) (1g) is dissolved in 10 ml dry 1,2-dichloroethane. The solution is cooled down to 10°C. Triethylamine (0,010 g, 5 mol %) is added to the solution of NCA. The reaction is followed by IR spectroscopy. After the end of the polymerisation the solution is precipitated in pentane, the product is isolated by filtration and dried under vacuum. The polymer is characterised by ^1H NMR (DMF-d_7). The molecular

weight is determined by ^{1}H NMR and GPC (polystyrene standards, THF as eluent). M_n = 25000. Yield 95 %.

6. Synthesis of poly-[N-(2-hydroxyethyl)-L-aspartate] with functional end group

Poly-[N-(2-hydroxyethyl)-L-aspartate] (PHEA) with functional end group(s) is synthesised by initiation of the polymerisation of TCEA-NCA with suitable initiators or by termination of the reaction with suitable terminating agents.

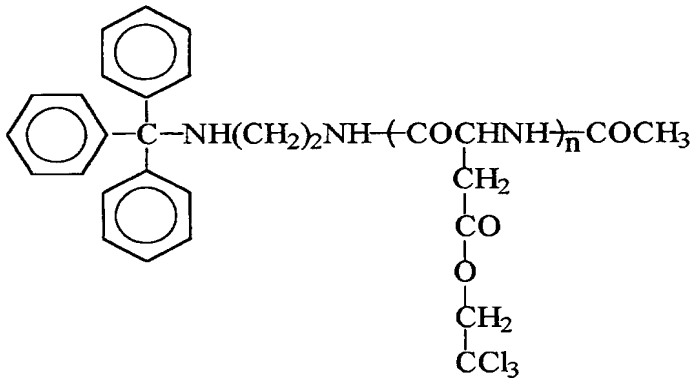
6.1. Synthesis of poly-[N-(2-hydroxyethyl)-L-aspartate] with amino end group

6.1.1. Polymerisation of N-carboxyanhydride of γ-trichloroethyl-L- aspartate by initiation with 1-triphenylmethylaminoethylamine

N-Carboxyanhydride of γ-trichloroethyl-L-aspartate (TCEA-NCA) (2 g) is dissolved in 20 ml dry 1,2-dichloroethane. The solution is cooled down to 10°C. 1-Triphenylmethylaminoethylamine (0,099 g, 5 mol % to TCEA-NCA) is dissolved in 2 ml 1,2-dichloroethane and added to the solution of NCA. After the end of the polymerisation, determined by IR spectroscopy, 3-fold molar excess of acetic anhydride and equimolar quantity of triethylamine are added and the reaction mixture is stirred for another 2 h at room temperature. The solution is precipitated in pentane and the polymer is isolated by filtration and drying under vacuum.

The molecular weight is determined by ^{1}H NMR (DMF-d_7) and GPC (polystyrene standards, THF as eluent). M_n = 5000. Yield 95 %.

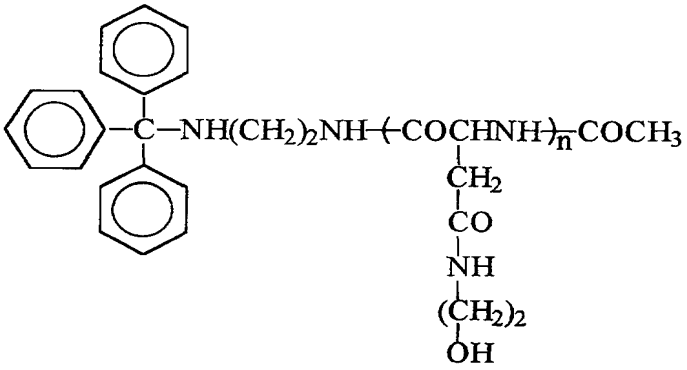
^{1}H NMR (DMF-d_7) analysis confirms the following structure of the polymer:



6.1.2. Aminolysis of trichloroethyl ester of poly-L-aspartic acid (6.1.1) with ethanolamine

1 g (3,8 mmol units) of the polymer is dissolved in 10 ml dry N,N-dimethylformamide. The solution is cooled down to10°C and 0,69 ml (11,5 mmol) ethanolamine and 0,36 g (3,8 mmol) 2-hydroxypyridine are added. The reaction is followed by IR spectroscopy. After the end of the aminolysis, the polymer is isolated by precipitation in ether, filtration and drying under vacuum. The product is purified by gel filtration on Sephadex G-25 (water as eluent) and isolated by lyophilization. The polymer is characterised by ^{1}H NMR (D_2O) and GPC (dextran standards, water as eluent). M_n = 3500. Yield 95 %.

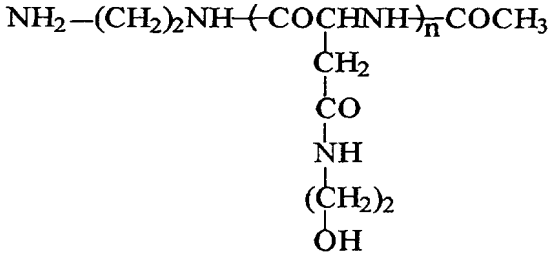
^{1}H NMR analysis confirms the following structure of the polymer:



6.1.3. Deprotection of poly-[N-(2-hydroxyethyl)-L-aspartate] (PHEA) with triphenyl-methyl end group (6.1.2)

1 g polymer is dissolved in 10 ml trifluoroacetic acid and stirred at room temperature for 0,5 h. Trifluoroacetic acid is removed by evaporation under vacuum. The polymer is dissolved in water and centrigugated. The supernatant is purified by gel filtration on Sephadex G-25 (water as eluent). The product is isolated by lyophilization.

^{1}H NMR (D_2O/DCl) analysis confirms the following structure of the polymer:



7. Coupling of aldehyde-terminated PHEG with human serum albumin

Aldehyde-terminated PHEG (2.5) in 0,1 M sodium acetate buffer(10 mg/ml), pH 4,0, is added to a solution of human serum albumin in the same buffer (10 mg/ml). After 16 h at room temperature the product is purified by gel filtration chromatography on Sephadex G-50 equilibrated with PBS. Fractions containing purified 1:1 PHEG-CH=N-HSA conjugates are pooled, concentrated by precipitation with ammonium sulfate, dissolved into PBS, and stored at 4°C.

When the coupling was carried out in the presence of a reducing agent, e.g. sodium cyanoborohydride, more stable cojugate, PHEG-CH_2NH-HSA is obtained.

8. Coupling of SS-Py terminated PHEG with human serum albumin

8.1. Thiolation of human serum albumin (HSA)

To HSA (10mg/ml) in PBS is added a 5-fold excess of SPDP, dissolved in minimal amount of DMF. After stirring at room temperature for 30 min, the solution is desalted into 0,1 M acetate buffer, pH 4,5, containing 0,1 M NaCl, and dithiothreitol (DTT) is added to give a concentration of 10 mM. After 20 min at room temperature, the DTT-treated mixture is desalted into 0,1 M sodium phosphate buffer, pH 7,2, containing1 mM ethylenediaminetetraacetic acid (EDTA). The number of thiol groups generated is determined with 5,5'-dithiobis(2-nitrobenzoic acid).

8.2. Reaction of SH-modified HSA with SS-Py terminated PHEG

For the preparation of a conjugate with disulfate bond, SS-Py terminated PHEG in 0,1 M sodium phosphate buffer, pH 7,2, containing1mM EDTA (10 mg/ml) is mixed with SH-modified HSA in the same buffer to give 4:1 molar ratio of HSA-SH over PHEG-SS-Py. After stirring at room temperature for 16 h, the conjugate is purified by gel filtration chromatography on Sephadex G-50 equilibrated with PBS. Fractions containing purified 1:1 HSA-SS-PHEG conjugates are pooled, concentrated by precipitation with ammonium sulfate, dissolved into PBS, and stored at 4°C.

The same conjugate could be prepared by the reaction of PHEG-SH with HSA-SPDP at similar conditions.

9. Coupling of maleimide-terminated PHEG with human serum albumin

9.1. Thiolation of human serum albumin

Thiolation of HSA is carried out a described in 5.1.

9.2. Reaction of SH-modified HSA with maleimide-terminated PHEG

For the preparation of a conjugate with thioether bond, maleimide-terminated PHEG in 0,1 M sodium phosphate buffer, pH 7,2, containing1mM EDTA (10 mg/ml) is mixed with SH-modified HSA in the same buffer to give 4:1 molar ratio of HSA-SH over PHEG-maleimide. After stirring at room temperature for 16 h, the conjugate is purified by gel filtration chromatography on Sephadex G-50 equilibrated with PBS. Fractions containing purified 1:1 HSA-S-PHEG conjugates are pooled, concentrated by precipitation with ammonium sulfate, dissolved into PBS, and stored at 4°C.

10. Biodegradation of poly[N-2-hydroxyethyl)-L-glutamines]

Poly(N-2-hydroxyethyl-L-glutamine) (PHEG) M_n 102000 (10 mg) is dissolved in 1,2 ml phosphate-citrate buffer, pH = 5,5, containing 0,2% (w/v) Triton X-100. 200 µl EDTA (10 ml in the buffer), 200 µl reduced glutathion (50 ml in the buffer) and 400 µl tritosomes or 100 µl cathepsine B (2,5 mg/ml in buffer) are added. The mixtures are incubated at 37 °C.
Samples are taken at predetermined periods of time and analysed by analytical GPC (dextran standards, phosphate-citrate buffer pH = 6,0 as eluent).
The results show decreasing of the molecular weight of PHEG, as an example :
at degradation with tritosomes M_n from 102000 to 12000 for 7 hr
at degradation with cathepsine B M_n from 102000 to 25000 for 7 hr.

Conclusions

The present invention provides poly alfa amino acid derivatives with functional end groups as well as the use and application of such polymers with reactive end groups to modify drugs, peptides, proteins and enzymes. The applicability of the present invention to modify drugs, peptides, proteins and enzymes is examplified by the modification of serum albumin as a

model protein but the present invention is not limited to serum albumin. The polymers with reactive end groups can be designed for and used for the coupling with either amino groups of proteins (eg. from lysine), carboxyl groups (eg from glutamic acid residues) or thiol groups (cysteine). An advantage of the present invention over PEO (see introduction) is that the PHEG-drived polymers in accordance with the present invention may be degradable by lysosomal enzymes. If the polymers or polymer-protein conjugates in accordance with the present invention would eventually end up in lysosomes they can be easily degraded. This is not the case with conventional PEO.

Claims

1. A method for preparing linear poly α-amino acid derivatives with one functional end group like an amine, alcohol, thiol, disulfide, carboxylic acid, carboxylic ester, vinyl, propenyl.....

2. A method for preparing linear poly α-amino acid derivatives having functional end groups at both chain ends, preferably these functionalities can be identical or heterogenous and be one of the following groups: amine, alcohol, thiol, disulfide, maleimide, carboxylic acid, carboxylic ester, vinyl, propenyl, styryl,.....

3. A method for preparing biodegradable poly[N-(2-hydroxy alkyl)-L-glutamines] with functional groups on either one or both chain ends.

4.A method for preparing poly[N-(2-hydroxy alkyl)-D-glutamines] with functional groups on either one or both chain ends.

5.A method for preparing poly[N-(2-hydroxy alkyl)-D,L-glutamines] with functional groups on either one or both chain ends.

6.A method for preparing biodegradable poly[N-(2-hydroxy alkyl)-L-aspartamine] with functional groups on either one or both chain ends.

7. End-group functionalized poly-α-amino acid derivative having the general formula

X - R1- Y -[- CO - CHR2 - NH -]n - Z

wherein
Z is selected from COCH3 , COC(CH3)=CH2 , CO(CH2)2COOH,
COOH and CO-R3-V wherein R3 is selected from polymethylene groups (CH2)n', n' being an integer from 2 to 20, aryl groups and alkylaryl groups, and V is selected from any of groups NH-A1, S-A2, O-A3,
CO-A4 and maleimido, A1, A2, A3 and A4 being protective groups respectively suitable for amine, thiol, alcohol and carboxyl,
Y is NH,
R1 is (CH2)m , m being an integer from 2 to 20,
X is an amino group selected from NH2, CHO , CH3O , (CH3O)2CH ,
NH-phenylmaleimide , NHC(C6H5)3 and NHCO(CH2)2-dithiopyridyl,
R2 is selected from (CH2)n-COO-CH2-CCl3 , (CH2)n-COO-CH2-CH3, (CH2)n-CONH-(CH2)n'-OH , e.g. (CH2)n-CONH-(CH2)3-OH and (CH2)n-CONH-CH2-CHOH-CH2OH,

n is an integer from 1 to 2,000.

8. An enzymatically degradable end-group functionalized poly-α-aminoacid derivative according to claim 7, being composed of L-aminoacid units.

9. A non enzymatically degradable end-group functionalized poly-α-amino-acid derivative according to claim 7, being composed of D-aminoacid units.

10. A method for preparing an end-group functionalized poly-α-aminoacid derivative according to claim 7, comprising ring-opening polymerization of an N-carboxy anhydride of γ-methyl, γ-benzyl or γ -trichloroethylester of L-glutamate, D-glutamate, L-aspartate or D-aspartate, characterized in that ring-opening polymerization is either initiated by means of a bifunctional initiator or terminated by means of a bifunctional terminating agent, thereby producing a poly-α-aminoacid derivative with a reactive group at one end of the polymer chain.

11. A method according to claim 10, characterized in that ring opening polymerization is both initiated by means of a bifunctional initiator and terminated by means of a bifunctional terminating agent, thereby producing a poly-α-aminoacid derivative with reactive groups at both ends of the polymer chain.

12. A method according to claim 10 or claim 11, characterized in that the said bifunctional initiator and/or the bifunctional terminating agent contains a primary amino group and a further functional protected group selected from amine, alcohol, carboxyl, ester, thiol, disulfide, maleimide, (meth)acrylate, (meth)acrylamide, vinyl, propenyl and styryl.

13. A method according to any of claims 10 to 12, characterized in that the bifunctional initiator and/or bifunctional terminating agent is 2-hydroxypyridine.

14. A method according to any of claims 10 to 13, characterized in that ring-opening polymerization of the N-carboxy anhydride is followed by aminolysis of the resulting polyglutamate or polyaspartate by means of 2-aminoethanol or 2,3-dihydroxypropylamine.

15. A method according to any of claims 10 to 14, characterized in that ring-opening polymerization is effected at a temperature between 0 and 40°C.

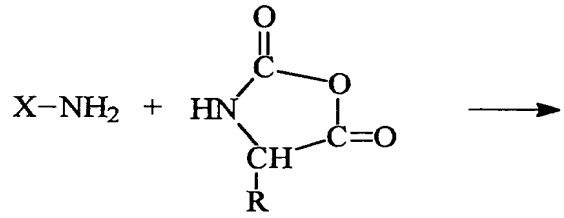
16 A method according to any of claims 10 to 15, characterized in that ring-opening polymerization is effected in a solvent for the N-carboxy anhydride.

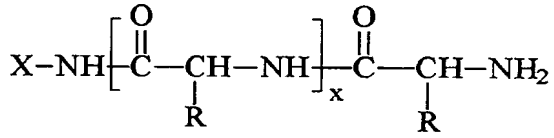
17 A method according to claim 16, characterized in that the said solvent is selected from 1,2-dichloroethane, methylene chloride, ethyl acetate, tetrahydrofuran and dimethylformamide.

18. Use of an end-group functionalized poly-α-aminoacid derivative according to any of claim 7 to 9 or of a product obtainable by a method according to any of claims 10 to 17 for the modification of a drug, a peptide, a protein or an enzyme.

19. The reaction product of coupling an end-group functionalized poly-α- aminoacid derivative according to any of claims 7 to9 with a drug, a peptide, a protein or an enzyme.

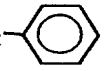
20. End-group functionalized poly-α-amino acid derivative as defined below as a compound or intermediate compound or manufacturable by the method defined below :

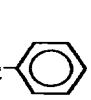




with R = $(CH_2)n-\overset{O}{\overset{\|}{C}}-OR'$

n = 1, 2

R' = CH_3, CH_2-  , CH_2CCl_3

X = A-NH-R" A = protective group : e.g. $-\overset{O}{\overset{\|}{C}}-O-CH_2-$ 

R" = $(CH_2)_n$, n = 2-20
aryl, aryl alkyl

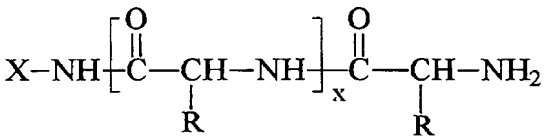
A-S-R" A = protective group

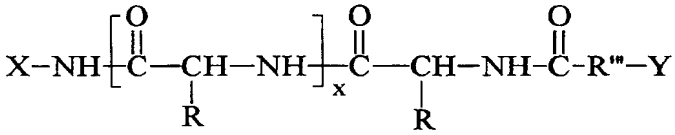
$-S-$  , trityl, ...

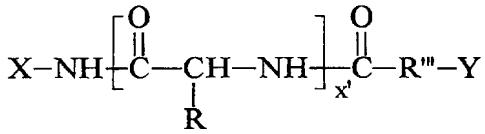
A-O-R" or

$A-\overset{O}{\overset{\|}{C}}-R"$

} A = protective group
e.g. tetrahydropyranyl, ...

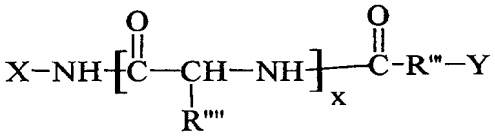






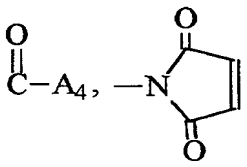
+ functional amine

e.g. H_2N-$(CH_2)_{n''}$-OH, $H_2N-CH_2-CH(OH)-CH_2OH$

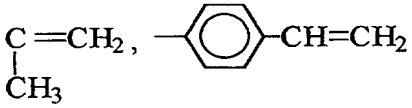


With $R''' = (CH_2)_{n'}$ $n' = 2\text{-}20$

Aryl, alkyl aryl,

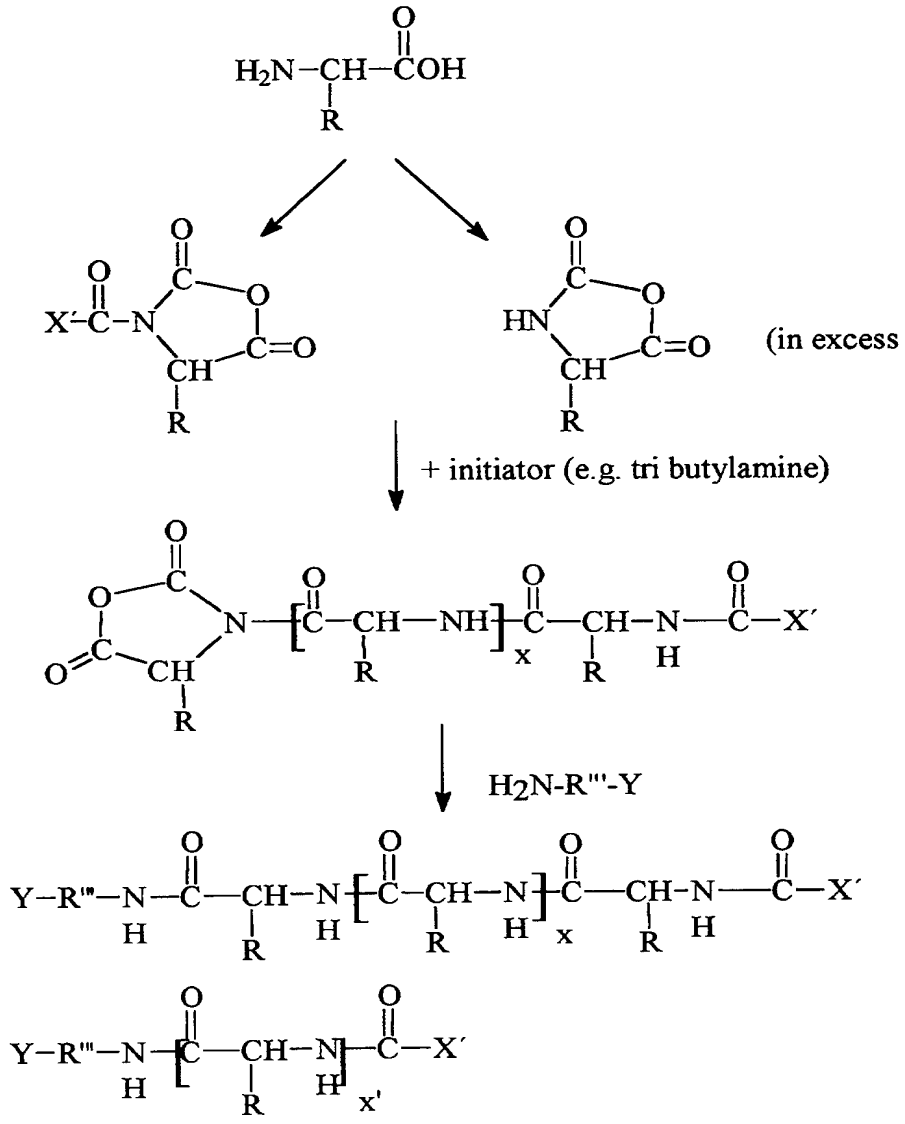
Y = NH-A_1, S-A_2, O-A_3, 

A = suitable protective group for amine (A_1), thiol (A_2), alcohol (A_3), carboxyl group (A_4)

or R''''-Y= $CH=CH_2$, 

$R'''' = (CH_2)n-C(=O)-NH-(CH_2)n''-OH$ or $(CH_2)_n$-CO-NH-CH_2-CHOH-CHOH

21. End-group functionalized poly-α-amino acid derivative as defined below as a compound or intermediate compound or manufacturable by the method defined below :

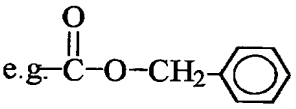


with R = $(CH_2)n-C(=O)-O-R'$ n = 1, 2

R' = CH_3, CH_2-(benzene), CH_2CCl_3

x′ is 1 to 2000, typically 500

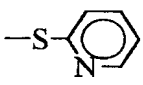
X′ = A-NH-R" or A-NH-R"-O-

A = protective group : e.g. 

R" = $(CH_2)_n$, n = 2-20
aryl, alkyl aryl

A-S-R" or A-S-R"-O-

A = protective group

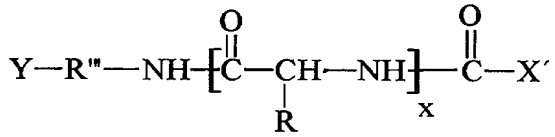
 , trityl, ...

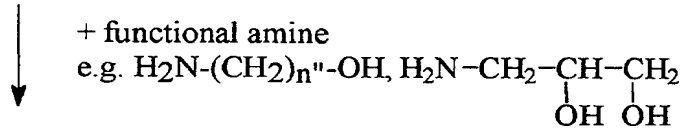
A-O-R"
or A-O-R"-O-
A–C(=O)–R"
or A–C(=O)–R"–O—

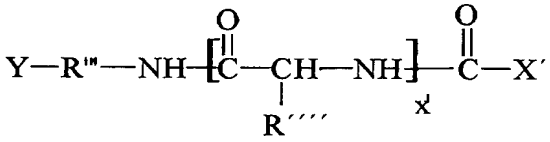
A = protective group
e.g. tetrahydropyranyl, ...

Y = R"-NH-A
R"-O-A
R"–C(=O)–A
R"-S-A

A = suitable protective group

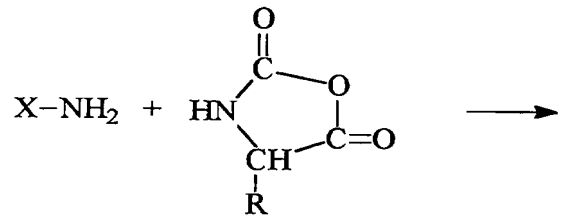


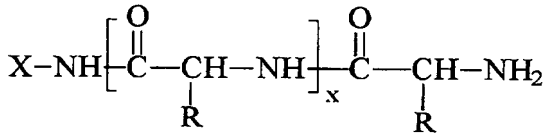




Where R′′′′ and R′′′ may be the same as for claim 20.

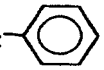
22. A method of manufacure of an end-group functionalized poly-α-amino acid derivative as defined below:





with R = $(CH_2)n-\overset{O}{\overset{\|}{C}}-OR'$

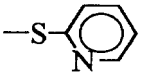
n = 1, 2

R' = CH_3, CH_2-  , CH_2CCl_3

X = A-NH-R" A = protective group : e.g. $-\overset{O}{\overset{\|}{C}}-O-CH_2$- 

R" = $(CH_2)_n$, n = 2-20
aryl, aryl alkyl

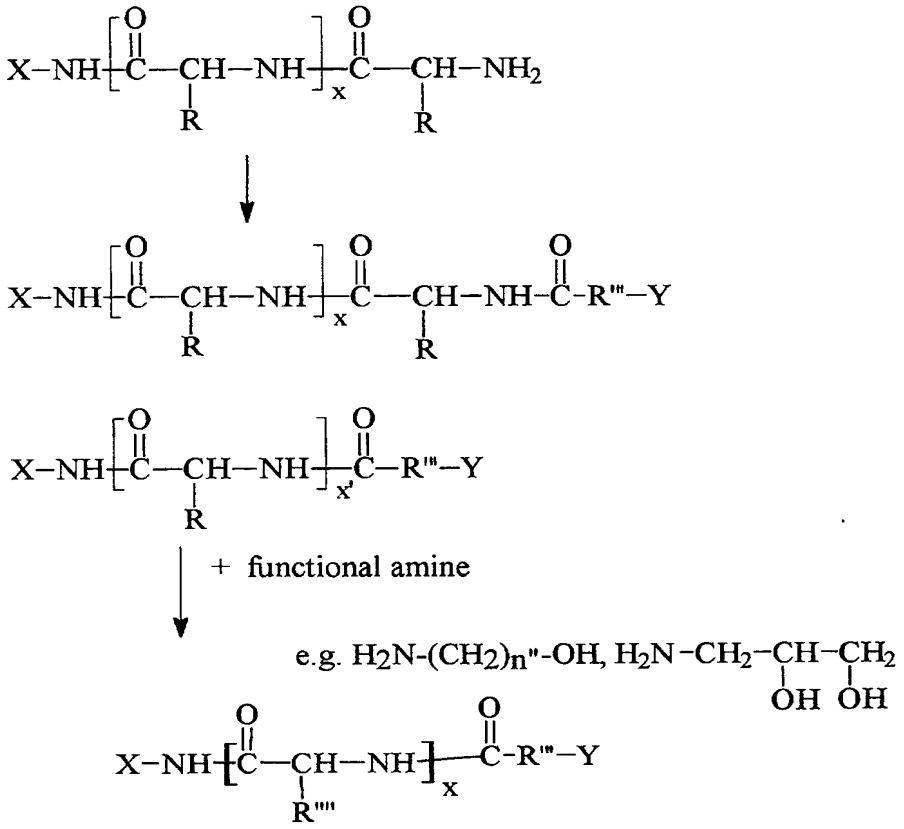
A-S-R" A = protective group

$-S$-  , trityl, ...

A-O-R" or

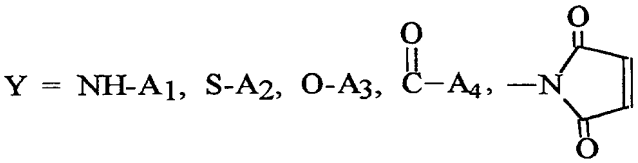
$A-\overset{O}{\overset{\|}{C}}-R"$

A = protective group
e.g. tetrahydropyranyl, ...

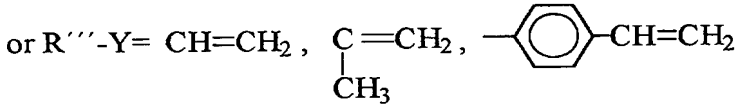


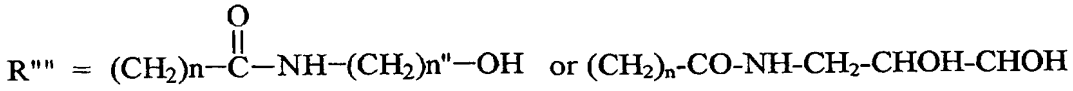
With $R''' = (CH_2)_{n'}$ $n' = 2\text{-}20$

Aryl, alkyl aryl,

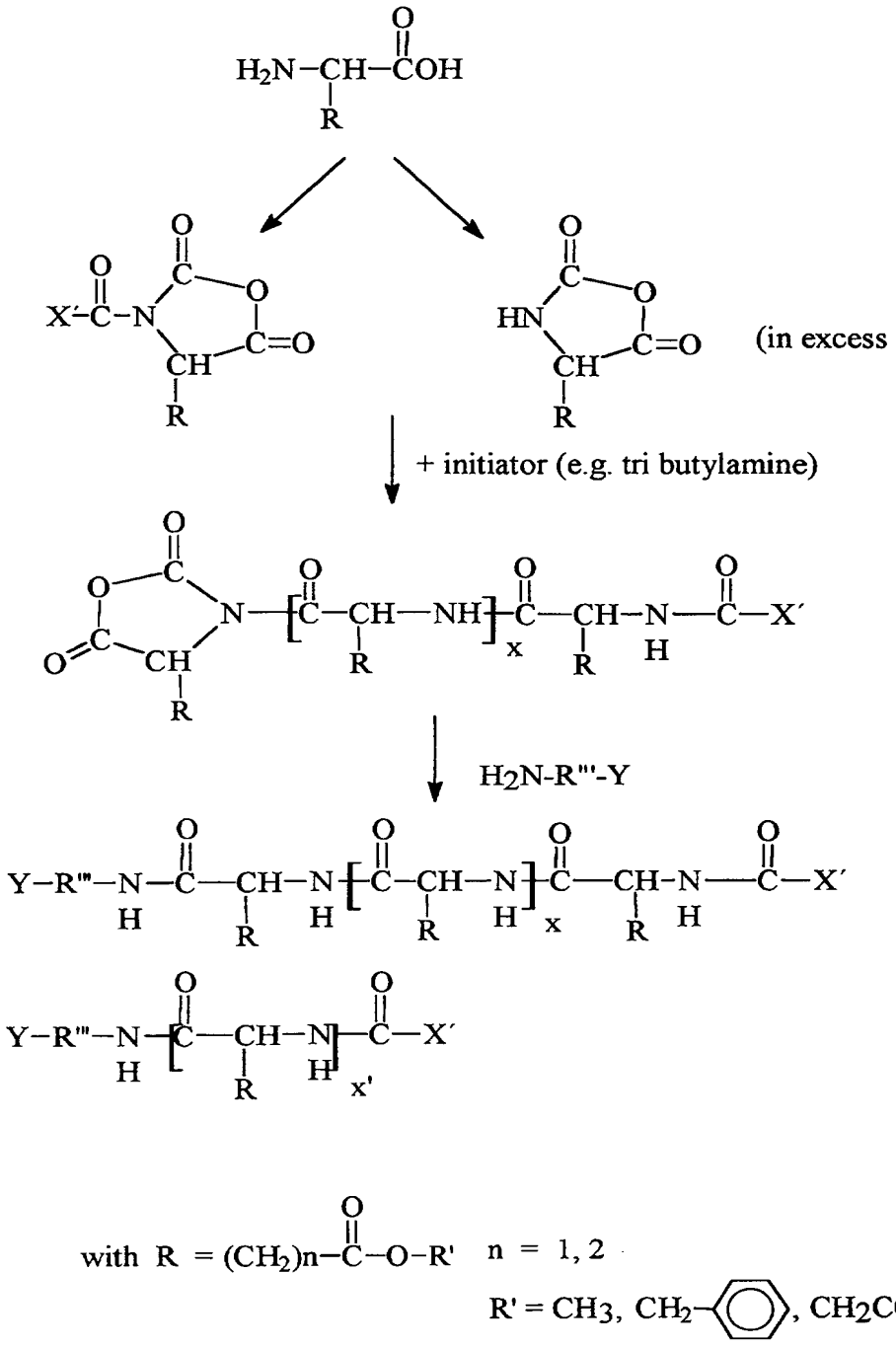


A = suitable protective group for amine (A_1), thiol (A_2), alcohol (A_3), carboxyl group (A_4)





23. A method of manufacure of an end-group functionalized poly-α-amino acid derivative as defined below:

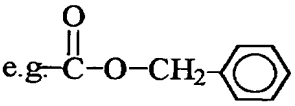


with R = $(CH_2)n-C(=O)-O-R'$ n = 1, 2

R' = CH_3, CH_2-(benzene), CH_2CCl_3

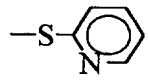
x′ is 1 to 2000, typically 500

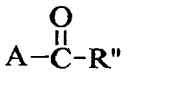
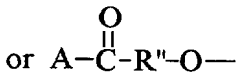
X′ = A-NH-R" or A-NH-R"-O-

A = protective group : e.g. 

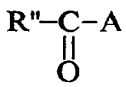
R" = $(CH_2)_n$, n = 2-20
aryl, alkyl aryl

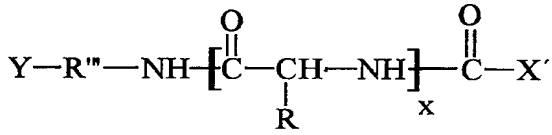
A-S-R" or A-S-R"-O-

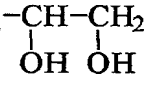
A = protective group
 , trityl, ...

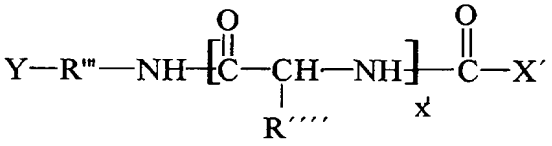
A-O-R"
or A-O-R"-O-



A = protective group
e.g. tetrahydropyranyl, ...

Y = R"-NH-A
R"-O-A

R"-S-A



+ functional amine
e.g. H_2N-$(CH_2)_{n"}$-OH, 



Where R′′′′ and R′′′ may be the same as for claim 22

24. Use of an end-group functionalized poly-α-aminoacid derivative according to claim 20 or 21 or of a product obtainable by a method according to claims 22 or 23 for the modification of a drug, a peptide, a protein or an enzyme.

25. The reaction product of coupling an end-group functionalized poly-α- aminoacid derivative according to claim 20 or 21 with a drug, a peptide, a protein or an enzyme.

26. A drug, a peptide, a protein or an enzyme including the end-group group functionalized poly-α-aminoacid derivative according to claim 20 or 21 or a compound or intermediate compound obtainable by a method according to claims 22 or 23.

27. An end-group functionalized poly-α-aminoacid derivative according to claim 20 or 21 or a product obtainable by a method according to claims 22 or 23 which is biodegradable.